COMPARISON OF NEUTROPHIL FUNCTION IN DIABETIC AND NON DIABETIC WITH GENERALIZED CHRONIC PERIODONTITIS AND IN GENERALIZED AGGRESSIVE PERIODONTITIS PATIENTS AND TO COMPARE THIS STUDY IN HEALTHY CONTROL GROUP

A Dissertation Submitted

in partial fulfillment of the requirements

for the degree of

MASTER OF DENTAL SURGERY

BRANCH – II

PERIODONTOLOGY

THE TAMILNADU DR. M.G.R. MEDICAL UNIVERSTIY
2008 – 2011

Certificate

This is to certify that **Dr. SUNIL D. PENDOR**, Post graduate student (2008 – 2011) in the Department of Periodontics, Tamilnadu Government Dental College and Hospital, Chennai – 600 003 has done this dissertation titled **"COMPARISON OF NEUTROPHIL FUNCTION IN DIABETIC AND NON DIABETIC WITH GENERALIZED CHRONIC PERIODONTITIS AND IN GENERALIZED AGGRESSIVE PERIODONTITIS PATIENTS AND TO COMPARE THIS STUDY IN HEALYHY CONTROL GROUP."** under our direct guidance and supervision in partial fulfillment of the regulations laid down by **The Tamil Nadu Dr. M.G.R. Medical University**, Chennai -600 032 for **M.D.S.,** (Branch – II) **Periodontology** degree examination.

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ACKNOWLEDGMENT

I express my deep sense of gratitude to **DR. K. MALATHI M.D.S.**, Professor and H.O.D., Department of Periodontics, Tamilnadu Government Dental College and Hospital, without her unstained guidance, support and encouragement, this study would not have been possible. Words cannot express my gratitude for her quiet confidence in my ability to do the study, her willingness to help clear the stumbling blocks along the way and her tremendous patience till the end of the study. I am also extremely grateful to **DR. S. KALAIVANI M.D.S.,** Professor, **DR. MAHEASWARI RAJENDRAN M.D.S.**, Associate Professors, Department of Periodontics, Tamilnadu Government Dental College and Hospital, Chennai – 600 003, for their expert guidance and moral support during the completion of this study. I consider myself privileged, to have studied, worked and completed my dissertation under them in the Department.

My sincere thanks to **DR. K.S.G.A. NASSER, M.D.S.,** Principal, Tamilnadu Government Dental College and Hospital, Chennai – 600 003, for his kind permission and encouragement.

I am extremely grateful to **DR.A.MUTHUKUMARASAMY, M.D.S., DR. M. JEEVA REKHA, M.D.S., DR.P.KAVITHA, M.D.S.**, Assistant professors, Department of Periodontics, Tamilnadu Government Dental College and Hospital, Chennai – 600 003 for their valuable suggestions, constant encouragement and timely help rendered throughout this study.

I thank to **DR. RAMA GOPALAN, M.D., Ph.D.,** Professor and HOD, Department of Pathology, **Institute of Basic Medical Science, Taramani, Chennai** for granting me permission to conduct this study in the Department.

I am extremely grateful to **DR. P. SHANTI, M.D., PH.D** Professor, Department of Pathology, **Institute of Basic Medical Science, Taramani, Chennai** for having taken special interest in my study and teaching me the basic of neutrophil function test.

I would like to express my very special gratitude to Mrs. Amidha, Technician, for helping me in my study.

I thank **DR.R. RAVANAN Msc.,M.Phil.,Ph.D.**,Associate Professor, Department of Statistics, presidency college ,Chennai,) for helping me with the statistics in the study.

I take this opportunity to express my gratitude to my colleagues and well wishers for their valuable help and suggestions throughout this study.

A special mention of thanks to all my patients for their consent, co-operation and participation in this study.

All glory and honour to **THE LORD ALMIGHTY** who gives me the strength to persist against all odds, whose loving kindness and mercies endureth forever.

My heartfelt and deep gratitude to all my family members, for their help, patience, love and prayers which have sustained me throughout this period.

ABSTRACT

BACKGROUND

Epidemiological studies have shown that the risk for periodontitis in diabetes mellitus patient is greater than non diabetics. Diabetes mellitus is now documented to significantly enhance susceptibility to severe periodontitis. Increased susceptibility to periodontitis has been associated with impaired neutrophil function in diabetes mellitus. The objectives of this study are to compare the neutrophil function in diabetic and non diabetic patients with generalized chronic periodontitis and in generalized aggressive periodontitis patients and to compare this study in control group.

AIM

To assess neutrophil function i.e. Chemotaxis, Phagocytosis and Specific Granule Release in Diabetic and Non Diabetic patients with Generalized Chronic Periodontitis and Generalized Aggressive Periodontitis in Indian population.

Materials and Method:

60 patients were selected to participate in the study which was divided into four groups. These patients were selected according to clinical and radiographic criteria. Blood samples from 60 patients were collected after drawing 5ml peripheral venous blood and then various assays were carried out to assess the neutrophil functions mentioned above. Statistical analysis of the results was done using **ANOVA** followed by **Tukey HSD** test.

Results:

It was found that defective chemotaxis present in diabetic and in generalized aggressive periodontitis patients. Defective phagocytosis was observed in diabetic, generalized chronic, and in generalized aggressive periodontitis patients. And defective specific granule release assay was observed only in diabetic patient when compared to healthy control.

Conclusion:

This study has proved that there is association between neutrophil dysfunction and severity of periodontal diseases. Hence, further longitudinal and clinical trials with larger sample size and newer sensitive techniques are needed.

DECLARATION

TITLE OF DISSERTATION	Comparison of neutrophil function in diabetic and non diabetic patient with generalized chronic periodontitis and in generalized aggressive periodontitis patients and to compare this study in healthy control group.
PLACE OF STUDY	Tamil Nadu Government Dental College & Hospital, Chennai-600003
DURATION OF THE COURSE	3 Years
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I hereby declare that no part of the dissertation will be utilized for gaining financial assistance/any promotion without obtaining prior permission of the Principal, Tamil Nadu Government Dental College & Hospital, Chennai-600003. In addition, I declare that no part of this work will be published either in print or in electronic media without the guide who has been actively involved in dissertation. The author has the right to reserve for publish of work solely with the prior permission of the Principal, Tamil Nadu Government Dental College & Hospital, Chennai-600003.

Head of the Department **Signature of the candidate**

CONTENTS

LIST OF ABBREVIATIONS

1.	AGE	Advanced glycated end products
2.	A.A.	Aggregatibacter Actinomycetemcomitans
3.	B	Bacteriods
4.	CAL	Clinical attachment level
5.	DM	Diabetes mellitus
6.	FMLP	Formyl methionyle leucine phosphate
7.	GCF	Gingival crevicular fluid
8.	GI	Gingival index
9.	GagP	Generalized Aggressive Periodontitis
10.	IDDM	Insulin dependent diabetes mellitus
11.	JP	Juvenile Periodontitis
12.	LJP	Localized Juvenile Periodontitis
13.	NBT	Nitro Blue Tetrazolium
14.	NADPH	Nicotine amide adenine-di-nucleotide phosphate (reduced)
15.	NIDDM	Non insulin dependent diabetes mellitus
16.	PMN	Polymorphonuclear leukocyte
17.	P. gingivalis	Porphyromonas gingivalis
18.	RPP	Rapidly progressing periodontitis

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INTRODUCTION

INTRODUCTION

Diabetes mellitus is a multifactorial disease. The disease is characterized by hyperglycemia, hyperlipidemia, and associated complication.[10, 18] Diabetes mellitus represents one of the major chronic health problem facing the world today. Diabetes mellitus develops from either defect in insulin production or an impaired utilization of insulin. Based upon these two conditions diabetes mellitus can be divided into two main types. Type I (IDDM) or juvenile diabetes caused by destruction of pancreatic islets cells. Type II (NIDDM) or adult onset diabetes which results from the defect in the insulin molecule or alter cell receptors for insulin and represents impaired insulin function rather than the deficiency. Periodontal disease is an infectious inflammatory process of multifactorial origin that involves the interplay between bacteria, host and environmental factors.[47] The dynamics between the host immune responses and oral bacteria is essential to understand the pathogenesis of periodontal diseases.[26]

Although numerous host defense mechanisms are called into an action by the bacterial invasion at the gingival sulcus, substantial evidence indicates that the Polymorphonuclear Neutrophils (PMNs) are the key cellular elements of the innate immune system, providing protection from invading bacteria.[48] Neutrophils impairment leads to increased susceptibility to Periodontitis in diabetic patient. Epidemiologic studies have shown that the risk for Periodontitis in diabetes mellitus patient is greater than non diabetic. Type I and II diabetes are significantly enhances susceptibility to severe Periodontitis. In Aggressive Periodontitis there is decreased responsiveness of PMN to chemotactic stimuli.[65] It has been demonstrated that their serum is shown to be a poor

source of chemotactic factor.[65] The important role of PMNs play in optimal functioning of the immune defense system, has led to speculation that a partly compromised system could severely weaken the defense mounted against a bacterial insult and permit the occurrence and progression of infections.[23] Since there are few studies which have been done in this context and very little data available in Indian population, the present study was undertaken to assess the invitro neutrophils chemotaxis, phagocytosis and specific granule release in diabetic and non diabetic patient with Generalized Chronic Periodontitis and in Generalized Aggressive Periodontitis patient.

AIM AND OBJECTIVE

AIM

The study was aimed to assess neutrophils function i.e. Chemotaxis, Phagocytosis and Specific Granule Release in Diabetic and Non Diabetic patients with Generalized Chronic Periodontitis and Generalized Aggressive Periodontitis in Indian population.

OBJECTIVES

1. To assess Chemotactic activity of neutrophils in Diabetic and Non Diabetic patients with Generalized Chronic Periodontitis and in Generalized Aggressive Periodontitis (GAgP) subjects.
2. To assess Phagocytic activity of neutrophils in Diabetic and Non Diabetic patients with Generalized Chronic Periodontitis and in Generalized Aggressive Periodontitis (GAgP) subjects.
3. To assess Specific Granule Release activity in Diabetic and Non Diabetic patients with Generalized Chronic Periodontitis and in Generalized Aggressive Periodontitis (GAgP) subjects.
4. To compare these neutrophil functions within the above forms of Periodontitis with the control group of periodontally healthy subject.

Review of Literature

REVIEW OF LITERATURE

DIABETES MELLITUS

Diabetes mellitus is a clinically and genetically heterogenous group of metabolic disorders manifested by abnormally high levels of glucose in the blood. The hyperglycemia is the result of a deficiency of insulin secretion caused by pancreatic beta cell destruction or of resistance to the action of insulin in liver and muscle or a combination of these. The current classification of diabetes is based upon the pathophysiology of each form of disease.[11]

Type 1 diabetes is one of the most frequent chronic childhood diseases. According to the American Diabetes Association, this form is present in the 5 -10% of patients with diabetes which is caused due to the pancreatic beta cell destruction as a result no insulin is produced.[11]

Type2 diabetes is the most common form of the disease. It has a stronger genetic component than type 1. It occurs because of a decreased responsiveness to insulin at the target organs. It typically begins at around middle age (40 years) and may be treated by dietary modification, oral hypoglycemic agents or may require insulin in uncontrolled cases. Patients with long standing diabetes frequently experience pathologic changes in many tissues and organs and the extent of diabetic complications is related to the degree of metabolic control.[10, 18]

DIAGNOSTIC CRITERIA FOR DIABETES MELLITUS [11]

1. Classical symptoms includes polyuria, polyphagia and polydipsia
2. Fasting plasma glucose level **≥126mg/dl (≥7.o m mol|L)**
3. Random or 2 hour post glucose loading venous plasma

   Glucose level of ≥ 200mg/dl (≥11.1 m mol\L).

INFLAMMATION AND DIABETES MELLITUS [11]

Inflammation is significantly pronounced in the presence of diabetes mellitus. Diabetics have impaired immune defense mechanisms which are more susceptible to infection and infections in diabetics are more severe when compared to non-diabetics. A bacterial infection decreases the effectiveness of insulin receptors on target tissue cells which reduces the ability of the body to utilize glucose. Poor metabolic control of diabetes results with a consequent increased risk of developing diabetic complications.

DIABETES – PERIODONTITIS – INTERRELATIONSHP [11]

The association between diabetes mellitus and Periodontitis has been the subject of much research and debate, with a myriad of research reports having produced conflicting results. Recent data suggests that Periodontitis may cause changes in systemic physiology. The inter relationship between Periodontitis and diabetes provides an example of systemic disease predisposing to oral infection and once that infection is established, the oral infection exacerbates the systemic disease. Both types of diabetes are risk factors for Periodontitis.

Joseph J Zambon et al (1988) [31] demonstrated that the sub gingival micro flora and serum antibody response was examined in Periodontitis patients with NIDDM. The predominant cultivable micro flora was determined for sub gingival plaque sampled from two deep periodontal pockets in each of 8 adult Periodontitis patients with NIDDM. Sub gingival plaque samples examined, demonstrated a high prevalence of black-pigmented bacteriods and suggested that the proportion of B. gingivalis (P. gingivalis) but not B. intermedia (P.intermedia) was higher in NIDDM with Periodontitis than in other groups.

Hugoson A et al (1989) [29] found that the prevalence and severity of periodontal disease in age and sex matched adults with long and short duration insulin dependent diabetics and non- diabetics were compared. The study involved 82 subjects with long duration and 72 short duration diabetics and 77 non-diabetics, all aged 20-70 years. Diabetics, irrespective of the duration of the disease, had a higher prevalence of sites with gingivitis than non-diabetics. Overall, there were no significant differences between the groups regarding the prevalence of tooth surfaces with probing pocket depths of 4 and 5mm. However, on comparison between age subgroups, long duration diabetics younger than 45 years had significantly more 4 and 5 mm pockets than non diabetics.

De Pommereau V et al (1992) [20] evaluated the periodontal status of 85, 12-18 year old French adolescents with IDDM and 38 healthy controls in the same age groups. Diabetic children had significantly more gingival inflammation than children without diabetes, in spite of similar plaque scores.

Erhan Firatli (1997) [22] compared the periodontal status of 44 insulin dependent diabetic children and adolescents and 20 healthy control subjects for a period of approximately 5

years. The differences is evaluated. The only statistically significant difference observed in the diabetic group was clinical attachment loss which was higher, compared to the controls and statistically significant. They concluded that diabetes modifies the clinical status of the periodontal tissues and increases the clinical attachment loss.

Rylander H et al (1997) [51] concluded in their studies that there were no significant correlations between the periodontal variables and the duration of diabetics or insulin dosage.

Paul A Moore et a1 (1999) [50] shows in a group of 320 adult dentate subjects (type I DM mean age 32.1) received a periodontal examination as part of the comprehensive oral health assessment Attachment loss was significantly greater for older patients whereas bleeding on probing and calculus levels were relatively constant across age categories

PERIODONTITIS

Periodontitis is an inflammatory disease that results in destruction and degradation of the soft and mineralized connective tissues that supports and houses the dentition. It is widely accepted that initiation and progression of periodontal disease are caused by the presence of pathogenic micro-organisms that invade the host. These microorganisms can cause direct damage to the periodontium, and they can also cause damage indirectly by inactivating a variety of host mediated pathways that result in connective tissue destruction.[2]

Neutrophils (Polymorphonuclear leucocytes [PMNs]) are critical components of the innate immune system and help to maintain oral health in the face of constant oral

bacterial challenge. PMNs are the first line of defence against a microbial challenge to the periodontium and serve a protective function through their ability to phagocytose and kill microorganisms. However, along with protecting the periodontium from microbial invasion, PMNs release potent lysosomal enzymes and oxygen radicals that can be destructive to the periodontal tissues.[2]

The vital role that PMNs play in protecting and maintaining the periodontium is evident in the oral manifestations present in patients with known qualitative and/or quantitative neutrophils disorders, such as benign neutropenia, Leukocyte Adhesion Deficiency type 1, and agranulocytosis. Although these diseases may have different clinical presentations and aetiologies, they are all associated with varying degrees of susceptibility to infection that manifest orally as severe Aggressive Periodontitis and the premature loss of primary and permanent dentition.[2]

Till now there have been several classifications proposed and none of them stood the test of time, because of their dependence on clinical presentation of disease alone. In 1999, the international workshop of a classification of periodontal diseases and conditions agreed upon new classification of aggressive periodontitis.[13]

AGGRESSIVE PERIODONTITIS is classified into

1. Localized

2. Generalized

PRIMARY features of **AGGRESSIVE PERIODONTITIS** are **(Lang et al 1999)**

1. Non-contributory medical history
2. Rapid attachment loss and bone destruction
3. Familial aggregation

SECONDARY features of **AGGRESSIVE PERIODONTITIS** are

1. Amount of microbial deposits inconsistent with the severity of periodontal destruction.
2. Elevated proportions of Aggregatibacter Actinomycetemcomitans and Porphyromonas Gingivalis
3. Phagocyte abnormalities
4. Hyper-responsive macrophage phenotype, including elevated production of PGE2 and IL-1ß in response to bacterial endotoxin.
5. Progression of attachment loss and bone loss may be self arresting

Features for Localized and Generalized Forms of Aggressive Periodontitis Are

LOCALIZED AGGRESSIVE PERIODONTITIS [3, 13]

1. Circumpubertal onset.
2. Localized first molar/ incisor presentation with interproximal attachment loss on at least two permanent teeth one of which is a first molar and involving no more than two other than first molars and incisors.
3. Poor serum antibody response to infecting agents.

GENERALIZED AGGRESSIVE PERIODONTITIS [3, 13]

1. Usually affecting persons under 30yrs of age, but patient may be older.
2. Generalized interproximal attachment loss affecting at least three permanent teeth other than first molars and incisors.
3. Pronounced episodic nature of the destruction of attachment loss and alveolar bone.
4. Poor serum antibody response to infecting agents.

MICROBIAL ETIOLOGY OF AGGRESSIVE PERIODONTITIS [54]

LOCALIZED AGGRESSIVE PERIODONTITIS

The most notorious relationship between specific bacterial infection and any periodontal disease is that between Aggregatibacter Actinomycetemcomitans and localized Aggressive Periodontitis.

1. Aggregatibacter Actinomycetemcomitans displays a number of virulent factors that would support its capacity to induce or propagate Aggressive Periodontitis.

2. Serological data indicates that the aggressive periodontitis patients can have very high antibody titers against Aggregatibacter Actinomycetemcomitans, indicating that they have been infected with the bacteria. As a group, Aggressive Periodontitis patients with such high antibody titers against Aggregatibacter Actinomycetemcomitans (and Porphyromonas Gingivalis) have less severe and extensive disease than do those who lack these antibodies.

3. A limited number of studies of treatment of localized Aggressive Periodontitis have shown that elimination of detectable levels of A.A. through modalities including scaling, root planning, surgery and antibiotic treatment resulted in cessation of attachment loss as well as diminution of antibody response.

4. A. Actinomycetemcomitans has been detected within diseased periodontal tissues in affected sites in localized aggressive periodontitis patients. Furthermore, the invasive properties of A. Actinomycetemcomitans can be demonstrated in invitro. Thus the data indicates that A. Actinomycetemcomitans is an important pathogen in localized aggressive periodontitis and likely to be responsible for a large percentage of associated lesions.

GENERALIZED AGGRESSIVE PERIODONTITIS

The bacteriology of Generalized Aggressive Periodontitis appears to be somewhat more complex than that of localized form. This may be due to the fact that the populations are more heterogeneous and thus could represent subpopulation with different aetiologies. The bacteria that are apparently the most representative of this group include Porphyromonas gingivalis and Aggregatibacter Actinomycetemcomitans as well as Prevotella Intermedia, Bacteriodes Forsythus, Eubacterium species, Fusobacterium Nucleatum, Campylobacter Rectus and number of spirochetes. Each is emphasized here because of its documented presence in the disease, induction of immune responses in human and virulent factors.

PMNs constitute one of the body's primary defences against invading bacteria or foreign entities. Drawn towards the latter by increasing gradients of the chemoattaractants

that the organisms generate the neutrophils task, once they reach them, to phagocytose and destroy the organisms. Their bactericidal functions lie in the contents of their granules, plasma membrane and cytoplasmic contents allows them to generate in or inject into the phagosome enclosing the organism.

The roles of chemoattractant and phagocytizable entities are very different and their effect on the neutrophils proceeds through different receptors and receptor mediated processes.

Neutrophils migrate to the sites of bacterial ingress or tissue damage through the process of chemotaxis. The term chemotaxis was introduced in 1884 by Pfeiffer, who described it as directional migration of leukocytes along a chemical gradient.[23] PMN chemotaxis can be stimulated by various N- Formylated peptides such as FMLP. These small peptides are of importance since they are thought to be structural analogs of bacterial products, and mimic the effect of these bacterial products on human PMN.[21] Other chemoattractants include C5a, a product of the complement cascade, products of phospholipids metabolism (leukotriene B4), and chemokines such as IL-8. The chemotactic response is initiated by the binding of the chemoattractant to specific PMN plasma membrane or to its cell surface receptor. This binding of chemoattaractants causes activation of the cytoskeletal machinery which in turn causes an array of cellular responses, including release of arachidonic acid and lysosomal enzymes and superoxide production as well as locomotion.[23]

Neutrophils chemotaxis is the first step of host defense and plays an important role in the host defence against bacterial infection. N-FMLP can elicit neutrophils chemotaxis to

the inflammatory site.[32] The Nitro Blue Tetrazolium (NBT) test measures the respiratory burst activity in PMN's by the reduction of NBT to formazan by the superoxide anion generated in the burst. It is an indicator of the degree of activity in the enzyme systems which are usually triggered by phagocytosis and which ultimately lead to bacterial killing. NBT tests have been described for peripheral blood PMN's however, healthy control values (unstimulated cells) always fall between 0-15% positive despite attention to technical detail.[52]

Clark, Page et al (1977) [14] evaluated the neutrophils chemotaxis in 9 patients with juvenile Periodontitis, 4 with normal subjects and 5 patients with the adult form of periodontitis as controls. Defective chemotactic responses were observed in Neutrophils from seven of nine juvenile patients, and a reduced level of complement-derived chemotactic activity was demonstrated in serum from four patients. These determinations were normal in all the patients with adult Periodontitis.

Cianciola LJ, Van Dyke TE et al. (1980) [62] Studied Polymorphonuclear leukocyte (PMNL) chemotaxis of 32 patients with localized juvenile Periodontitis (LJP), 10 adult patients with a history of LJP (post-LJP), 8 patients with Generalized juvenile Periodontitis (GJP), and 23 adults with moderate to severe Periodontitis. Based upon statistical analysis of chemotaxis assays, most carried out on at least two and often three or more separate occasions, 26 of 32 LJP patients, 7 of 10 post-LJP patients, and 5 of 8 GJP patients exhibited cellular defects of chemotaxis, whereas only 2 of 23 of the patients with adult Periodontitis exhibited depressed chemotaxis. Elevated PMN chemotaxis was occasionally found in subjects with juvenile Periodontitis (2 of 32 with LJP and two of eight with GJP); however, it was found in a significant number (10 of 23)

of patients with adult Periodontitis. The results indicate that the PMNL chemotaxis defect observed in juvenile Periodontitis is due to a cell-associated defect of long duration.

Van Dyke TE, Levine MJ et al. (1983) [63] studied the binding of the chemotactic complement fragment, C5a, to peripheral blood neutrophils of Localized Juvenile Periodontitis (LJP) patients and normal controls which was quantitated using iodinated human C5a and a rapid centrifugation assay. They observed a significant reduction in the number of binding sites per cell on neutrophils from the patient group, whereas the binding affinity remained the same as control values.

Suzuki, Neumann et al. (1983) [56] examined peripheral blood neutrophils from 29 LJP, 24 GJP and 24 healthy subjects and assessed for chemotaxis, phagocytosis and spore germination. Chemotaxis assay was performed using Boyden diffusion chamber using 10-8 M N-FMLP as chemoattractant and found that 23 of 29 LJP and 14 of 25 GJP subjects had a neutrophils defect.

Phagocytosis assays were performed in vitro using radiolabel bacterial spores Bacillus cereus. 18 of 29 LJP and 7 of 24 GJP had a phagocytic defect. Neutrophils induced spore germination assessed in vitro showed that 13 of 20 LJP and 3 of 8 GJP had this defect.

Ellegaard Borregaard et al. (1984) [8] Performed quantitative studies on phagocytosis on peripheral blood neutrophils isolated from 12 young and 10 adult patients with advanced Periodontitis. Chemotaxis was significantly reduced only in the young patients whereas no difference could be demonstrated between young and adult patients or controls in uptake of opsonised zymosan particles, in the concentration and consumption rate of

ATP, in the rate of oxygen consumption and oxygen liberation and lysozyme during phagocytosis.

Page RC, Altman LC et al. (1985) [48] examined cell motility of PMNs and MNs from 27 patients with rapidly progressive Periodontitis, 5 patients with juvenile Periodontitis, and 37 normal control subjects by using a micro chamber technique and the synthetic peptide N-formyl-methionyl-leucyl-phenylalanine (FMLP) as the chemoattractant. As a group, PMNs and MNs from patients with rapidly progressive Periodontitis manifested significantly enhanced random migration relative to control cell suppressed directed migration (chemotaxis) at FMLP doses of 10(-9) and 10(-8) M and enhanced directed migration at a dose of 10(-6) M FMLP. In contrast, PMNs from patients with juvenile periodontitis exhibited normal random migration, and directed migration was significantly suppressed at all doses of FMLP tested. An abnormality of either PMN or MN motility was observed in 26 of 27 patients with rapidly progressive Periodontitis. Enhanced random migration was seen in PMNs in 63%, MNs in 39%, and both cell types in 26% of the patients. Suppressed chemotaxis was seen in PMNs in 85%, in MNs in 74%, and in both cell types in 69% of the patients. Thus, most, if not all, of this subgroup of patients with early onset, highly destructive Periodontitis have abnormalities in PMN or MN motility, and these defects may differ from those seen in cells from patients with the juvenile form of the disease.

S. Singh, Golub et al. (1985) [57] developed an in vivo assay to monitor the crevicular leucocyte response to chemotactic agents, e.g., casein and N-formyl peptides. They included a control group with little or no gingival disease (C group), gingivitis (G group), chronic periodontitis (CP group) and Localized Juvenile Periodontitis (LJP group).

Casein (2mg/ml) was placed into an isolated gingival crevice of each subject with a calibrated wire loop and the time was recorded (t=0). Leucocytes were counted in crevicular washes 15 minutes later and every 5 minutes thereafter up to t=50 minutes. This protocol was repeated for the crevice of an adjacent tooth except that the crevicular fluid response to the chemotactic challenge was monitored. The C,G,CP group showed a similar pattern of response to the chemoattractant with a single "peak" of leucocytes at approximately t=25 minutes. LJPs showed an abnormal pattern with two leukocytes peaks, one at approximately 25 minutes and the other at 45 minutes.

Taufiq A, Offenbacher S, Van Dyke TE. (1986) [64] evaluated chemotaxis, phagocytosis, specific granule release and superoxide production in a group of 23 previously unreported LJP patients. Both granule release and superoxide production were found to be normal in chemo tactically defective LJP patients.

Cogen, Roseman et al. (1986) [53] compared 13 LJP, 5 GJP patients and their matched controls with respect to selected leukocyte functions and indicated that there were significant decreases in the phagocytic and chemotactic abilities of PMN in both LJP and GJP. All JP patients displayed intrinsic cell defects in chemotaxis compared with controls and some patients displayed multiple defects including those which were serum associated.

Page, Beaty et al. (1987) [49] observed that both neutrophils and monocytes from patients with generalized prepubertal periodontitis do not adhere to surfaces normally, and this defect is thought to be responsible for the observed abnormality in chemotaxis.

Kinane DF. Cullen. (1989) [33] The locomotory behaviour of peripheral blood neutrophils (PMNs) from patients with juvenile (JP) and rapidly progressive (RPP) forms of early-onset periodontal disease was studied under agarose technique and formyl methionyl-phenylalanine (FMLP) as the chemoattractant. PMNs from experimental patients showed normal random, chemotactic and chemokinetic locomotory behaviour when compared with control subjects. Further investigation of single-cell movements using time-lapse video analysis also failed to show any significant differences in locomotory behaviour between the PMNs of experimental and control individuals. They concluded that the differences in technique may account for much of the variation which exists in the literature with respect to PMN locomotion in periodontal disease. In the final analysis, it is difficult to dispute direct observation of moving cells and using this approach, they have been unable to confirm the presence of any PMN locomotory defect in their series of patients with early-onset periodontal disease.

Heikki Repo, Saxen et al. (1990) [27] studied the chemotaxis of peripheral blood polymorphonuclear leukocytes (PMNs) and monocyets and the production of tumour necrosis factor alpha by monocytes of patients with juvenile Periodontitis (JP). As a group, the patients' PMNs showed significantly increased chemotaxis determined by counting the number of migrating cells within a 3um-pore-size filter. Determined as distance of migration within the filter, as chemotactic increment based on checkerboard analysis, as leukotactic index calculated on the basis of distance of migration and cell count at different depths within a 3-µm-pore-size filter, as distance of migration under agarose, and as the number of PMNs migrating across a 5-µm-pore-size filter, the chemotactic migration rates of PMNs of patients were similar to those of controls.

Evaluation of the data on an individual basis suggested that in terms of PMN Chemotaxis, some patients were hyper responsive and some were hypo responsive. Chemotaxis, spontaneous migration and the rates of lipopolysaccharide-induced Tumour Necrosis Factor Alpha production by localized aggressive monocytes were similar to those of control cells.

Zafiropoulos, Flores et al. (1991) [71] examined the oxidative metabolism of PMNs in 19 RPP, 10 LJP, 10 AP and 39 healthy control subjects and was compared using the luminal chemiluminiscence (CL) method. In all groups, CL was significantly higher with autologous serum than with normal pooled serum and there was a significant linear relationship between the two values. There was a serum induced defect in 2 patients and 1 control.

Schenkein, Best et al. (1991) [58] examined chemotaxis of PMNs from 492 subjects with various periodontal diagnoses and found that there was an influence of race on the chemotactic response of periodontally healthy individuals whether or not they were related to patients with Early Onset Periodontitis**.**

Ashkenazi, White et al. (1992) [4] carried out a study to determine if an extract of A. Actinomycetemcomitans could induce changes in PMN chemotaxis and showed that when neutrophils were preincubated with the bacterial extract, chemotaxis toward zymosan-activated serum, FMLP and the bacterial extract was inhibited in two different chemotaxis assays i.e. Boyden chamber and under agarose assay and suggested that A. Actinomycetemcomitans may contribute to the pathogenesis of localized aggressive by inhibiting chemotaxis.

Biasi D, Bambara et al. (1999) [9] evaluated neutrophil function in patients suffering from the generalized form of early onset periodontitis (EOP). They concluded that neutrophils function in patients suffering from early onset periodontitis does not differ from control subjects, suggesting that the overall defence function of these cells are normal.

Kobayashi T, Sugita N et al. (2000) [35] evaluated whether FcγR polymorphisms are associated with generalized early onset Periodontitis in Japanese patients. Thirty eight Japanese patients with G-EOP and 83 Japanese patients with aggressive periodontitis were included in study. FcγR genotypes for 3 bi-allelic polymorphisms were determined in these generalized early onset periodontitis and adult periodontitis and 104 races-matched healthy controls by means of allele's specific polymerase chain reaction. There was a significant difference in the distribution of FcγRIIIb genotypes between G-EOP patients and healthy controls. The study indicates that FcγRIIIb-NA2 alleles could be associated with susceptibility to generalized early onset Periodontitis.

Kazuyuki Shibata, Warbinton et al. (2001)[36] examined Nitric Oxide Synthatase (NOS) involvement in chemotaxis of normal neutrophils and NOS activity in neutrophils from 10 normal subjects and 10 LAgP subjects .peripheral venous blood was isolated and membrane associated NOS and soluble NOS were extracted from cells with or without FMLP stimulation. NOS activity was measured using the radiolabeled L-arginine to L-cit-rulline conversion assay and they suggested that NOS is present in human neutrophils and may be involved in FMLP induced chemotaxis in normal neutrophils and NOS activity is increased in LAgP and is negatively correlated to chemotaxis response.

Loos et al. (2003) [39] evaluated the frequency of FcγRIIIb in 21 aggressive periodontitis patients and 26 healthy controls and found that FcγRIIIb-NA1/NA1 genotype in aggressive patients was significantly higher than in healthy control.

Chung H-Y et al. (2003) [15] done a case-control study in which FcγRIIa, FcγRIIIa, FcγRIIIb in 32 aggressive patients, 72 chronic patients and 72 race-matched healthy controls in Japanese individuals. There was a significant over-representation of the FcγRIIIb-NA2 allele and FcγRIIIb 232 THr in the aggressive periodontitis group as compared with healthy control groups. No differences in FcγRIIa, FcγRIIIa genotype/allele distribution were demonstrated between the two groups.

Nibali L et al. (2006) [44] done a case-control association study to test the association of specific gene polymorphisms affecting PMN functions with aggressive periodontitis. A blood sample was collected from subjects and genotypes $p22^{phox}$ (CYBA) and FcγR were analyzed in a blind fashion. Results showed concomitant presence of C242T $p22^{phox}$ NADPH oxidase T allele and FcγRIIIb-NA1 homozygosity was associated with generalized Aggressive phenotype in Caucasians. C242T $p22^{phox}$ NADPH oxidase and FcγR polymorphisms may predispose to Aggressive Periodontitis through modulation of neutrophils superoxide production.

A M Johnstone, Koh et al. (2007) [5] examined neutrophils function of 12 non-smoking patients who had been diagnosed with refractory Aggressive Periodontitis (RAP), 10 patients with chronic Periodontitis (CP), and 13 periodontally healthy controls (HCs). Their findings demonstrated a larger receptor independent respiratory burst and higher

phagocytic activity in PMNs derived from patients with RAP compared to PMNs derived from CP patients and periodontally HCs.

Shetty, Thomas et al. (2008) [45] evaluated neutrophils functions i.e. chemotaxis, superoxide production, phagocytosis and killing of Porphyromonas Gingivalis in 30 diabetics and 30 non diabetic patients with chronic generalized Periodontitis relative to healthy and matched controls. Their analyses revealed a significant depression in the number of diabetic PMNs migrating along an FMLP gradient, significant enhancement of diabetic PMN superoxide production was observed and phagocytosis and killing by diabetic PMN of P. gingivalis was also impaired significantly.

Carvalho, Mesquita et al. (2009) [16] evaluated the correlation between PMN phagocytosis and oxidative burst with the sub gingival microbiota of patients with Generalized Aggressive Periodontitis (GAgP). They concluded that GAgP subject presented diminished phagocytic activity of peripheral PMNs and high prevalence and levels of classical periodontal pathogens. No differences between groups were found for superoxide production.

MATERIAL AND METHOD

MATERIALS AND METHOD

SOURCE OF DATA:

This study was carried out on patients selected from the Out Patient Department, Department Of Periodontology, **Tamilnadu Government Dental College and Hospital**, Chennai. The study was carried out after the institutional ethical committee approval. The clinical material for the study consisted of 60 patients of either sex ranging in age between 13 - 60 years.

INCLUSION CRITERIA:

- Number of teeth present should not be less than - 20
- Number of sites involved should have ≥ 2mm clinical attachment loss with presence of disease activity as recorded by Gingival index **(LOE AND SILNESS)**
- Full mouth intraoral periapical radiographs were taken.

EXCLUSION CRITERIA:

- History of any systemic disease for the control group.
- History of any systemic disease other than diabetes for the test group
- Brittle diabetics.
- Pregnant women and lactating mothers
- Presence of any habits like smoking and alcoholism
- History of antibiotic therapy within 6 months prior to study.

SUBJECT STRATIFICATION:-

60 patients in the age group varying between 13-60 years from both sexes were selected.

60 patients were divided into four groups:-

Group I – 15 Diabetic with Generalized chronic Periodontitis patients.

Group II – 15 Non Diabetic with Generalized chronic Periodontitis patients.

Group III – 15 Generalized Aggressive Periodontitis patients.

Group IV - 15 Control group of periodontally healthy patients

SELECTION OF THE PATIENTS

All the patients selected for this study were subjected to an intraoral examination under artificial light using a mouth mirror and William's probe. Plaque index **(SILNESS AND LOE 1964)** and **RUSSELL'S PERIODONTAL INDEX (1956)** were used for the assessment of plaque and the periodontal condition of the patient.

CRITERIA FOR SELECTION

GROUP I - DIABETIC WITH GENERALIZED CHRONIC PERIODONTITIS [3]

Patients selected for this group on the basis of the following features:

1. Age 13 to 60 years
2. Fasting plasma glucose should be equal or greater than 126mg/dl.
3. Generalized alveolar bone loss with multiple horizontal and vertical osseous defects.

GROUP II – NON DIABETIC WITH GENERALIZED CHRONIC PERIODONTITIS [3]

1. Age 30 -35 years or more
2. Amount of microbial deposits is consistent with the severity of periodontal tissue Destruction
3. Generalized severe alveolar bone loss with multiple horizontal and vertical osseous defects.
4. Disease usually affects many teeth with no rapid progression of the disease

GROUP III – GENERALIZED AGGRESSIVE PERIODONTITIS [13]

1. Include patients under 30 years of age or older,
2. Pronounced destruction of alveolar bone and generalized attachment loss affecting three permanent teeth other than first molar and incisors.
3. Amount of microbial deposits is inconsistent with the severity of periodontal tissue destruction.
4. Except for the presence of Periodontitis, patients are otherwise clinically healthy.

.

GROUP IV - CONTROL GROUP

Periodontally healthy subjects who had Plaque and Russell's Periodontal Index score 0 were included in this group.

A standard proforma consisting of the following data: Name, age, sex, past medical and dental history, plaque index (**SILNESS AND LOE 1964**) and **RUSSELL'S**

PERIODONTAL INDEX (1956) and clinical attachment loss for the each patient was recorded.

PLAQUE INDEX (Pl) (SILNESS AND LOE 1964) [59]

Index used for assessment of plaque. It assesses only the thickness of plaque at the gingival area of the tooth. The surfaces examined are the four gingival areas of the tooth i.e. the disto-facial, facial, mesio-facial and lingual surfaces. The teeth and the gingiva were air dried and examined with mouth mirror and a dental explorer. Scoring criteria used is as follows:

0 – The gingival area of the tooth surface is literally free of plaque. The surface is tested by running a pointed probe across the tooth surface at the entrance of the gingival crevice after the tooth has been perfectly dried. If no soft matters adhere to the point of probe, the area is considered clean.

1 – No plaque can be observed in situ by the naked eye. A film of plaque adhering to the free gingival margin and adjacent area of the tooth which can be recognized only by running the explorer/pointed probe across the tooth surface or by using a disclosing agent.

2 – A thin to moderate accumulation of soft deposits within the gingival pocket or on the tooth and gingival margin, which can be seen with the naked eye.

3 – Abundance of soft matter within the gingival pocket and or on the tooth surface and gingival margin. The interdental area is stuffed with soft debris.

Plaque index (Pl I) for a tooth – scores from four areas of tooth

Nominal scale for patient evaluation:

SCORES	RATING
0	Excellent
0.1-0.9	Good
1.0-1.9	Fair
2.0-3.0	Poor

PERIODONTAL INDEX (PI) (RUSSELL 1956) [59]

The PI was intended to estimate deeper periodontal disease by measuring the presence or absence of gingival inflammation and its severity, pocket formation, and masticatory function.

All the teeth present are examined. All of the gingival tissue circumscribing each tooth is assessed for gingival inflammation and periodontal involvement.

Calculation of the index:

$$\text{PI score per person} = \frac{\text{sum of individual scores}}{\text{Number of teeth present}}$$

PI scoring criteria :

SCORE	CRITERIA	X-RAY CRITERIA
0	**NEGATIVE:** there is neither overt inflammation in the investing tissues nor loss of function due to destruction of supporting tissues.	Radiographic appearance is essentially normal.
1	**MILD GINGIVITIS**: there is an overt area of inflammation in the free gingivae, but this area does not circumscribe the tooth.	
2	**GINGIVITIS**: inflammation completely circumscribing the tooth, but there is no apparent break in the epithelial attachment.	
4		There is early, notch like resorption of alveolar crest
6	**GINGIVITIS WITH POCKET FORMATION**: the epithelial attachment has been broken and there is a pocket. There is no interference with normal masticatory function, the tooth is firm and has not drifted.	There is horizontal bone loss involving the entire alveolar crest, up to half of the length of the tooth root.

8	**ADVANCED DESTRUCTION WITH LOSS OF MASTICATORY FUNCTION**: the tooth may be loose, may have drifted, may sound dull on percussion with a metallic instrument, may ne depressible in socket.	There is advanced bone loss, involving more than one half of the length of tooth root, or a definite infrabony pocket with widening of periodontal ligament. There may be root resorption or rarefaction at apex.

Clinical conditions and periodontal scores:

CLINICAL CONDITION	**GROUP PI SCORES**	**STAGE OF DISEASE**
Clinically normal supportive tissues	0 – 0.2	
simple gingivitis	0.3 – 0.9	Reversible
Beginning destructive periodontal disease	0.7 – 1.9	Reversible
Established destructive periodontal disease	1.6 – 5.0	Irreversible
Terminal disease	3.8 – 8.0	Irreversible

COLLECTION OF BLOOD

ARMAMENTARIUM

- Disposable hypodermic syringes
- 23 gauge needle
- Sterile vials
- Tourniquet
- Cotton swabs
- Spirit

Skin preparation was done and 5 ml of venous blood was drawn from the anticubital vein with a needle and a disposable syringe; 2.5 ml of the blood was transferred into a plain vial (for phagocytosis) and the remaining 2.5ml into the vial containing Heparin and transported immediately to the laboratory in a box containing ice pack.

TESTING FOR NEUTROPHIL FUNCTIONS [41, 46, 43, 60, 71]

The patients selected for the present study underwent the following neutrophils function tests

1. CHEMOTAXIS

2. PHAGOCYTOSIS

3. SPECIFIC GRANULE RELEASE ESTIMATION**.**

NEUTROPHIL CHEMOTAXIS ASSAY

DETAILS OF TECHNIQUE:

UNDER AGAROSE TECHNIQUE [41, 46]

Migration of neutrophils in response to chemoattractant in the environment is carried out by using viable cells suspended in a balanced salt solution (Hank's) or Minimal Essential Media (MEM) using agarose technique.

PREPARATION OF CELLS

Blood was drawn into sterile disposable syringe with 10 -20 unit of Heparin per ml of blood erythrocytes were sedimented by gravity at 37^0c. The blood was centrifuged at 2000 RPM for 30 minute and polymorphs obtained.

EQUIPMENT

1) 37°C Incubator

2) A means for low power magnification

REAGENTS:

1.2G/ml agarose

120 mg agarose powder was dissolved in 5ml distilled water by heating it 10-15 min and cooled to 48^0C for

SUPPLEMENTED

a) 100 µl of 10x MEM

b) 100 µl of heat inactivated pooled human serum

c) 100 µl 7.5% sodium bicarbonate

d) 2.9 ml distilled water

3. 10^{-8} M FMLP as chemoattractant was used

STAINING REAGENT used were

A) 2 to 3 ml methanol

B) 2 to 3 ml formalin

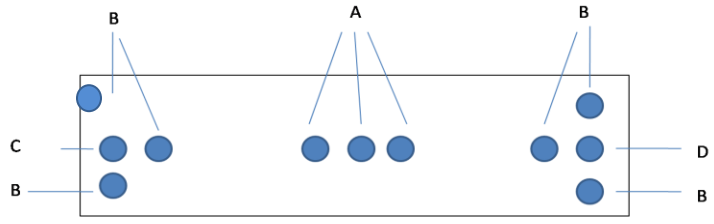
C) Wrights stain

PREPARATION OF AGAROSE PLATE

120 mg of agarose powder was dissolved in 5ml distilled water by heating it for10-15 minutes and cooled to 48^0c. The agarose was mixed with an equal volume of prewarmed MEM and fetal calf serum mixture whose p^H was brought to alkalinity with sodium bicarbonate. 6 ml of the agarose medium was layered onto the rectangular glass slide and allowed to harden for half an hour to facilitate the cutting of wells.

PREPARATION OF AAROSE WELLS

1. Series of three wells 3mm in diameter were cut and spaced 3mm apart for this purpose and 11 gauge punches such as Pasteur pipette attached to a vacuum was used
2. Arrangement of wells for agarose chemotaxis assay



a. FMLP chemoattaractant

b. White blood cells

c. MEM

d. Serum

METHOD FOR CHEMOTAXIS

1. 0.01ml of FMLP chemoattaractant was added to A well
2. 0.01ml of WBC was added to B well
3. 0.01ml MEM was added to C well
4. 0.01 ml serum was added to D well

PMN's were incubated for 37^0C for 2 hours. After incubation, cells were fixed and stained which are as follows:

a. Culture plates were flooded with 3-5 ml methanol for 30 min and removed.

b. 3 -5 ml formalin was flooded for 30 min.

c. Agarose was removed.

d. WBC's were stained on bottom of culture plates with Wright's stain for 15 min and washed with distilled water.

CALCULATION:

1. Culture plates were projected under the microscope.
2. Chemotaxis was measured as linear distance (in cm) that WBC cells (from B) have migrated from margin of well toward FMLP chemoattractant (A).

PHAGOCYTOSIS ASSAY: [43, 60]

Suspension of killed spore's Candida albicans was incubated with buffy coat preparation of leukocytes. The number of Candida spores internalized by each phagocyte was then counted under the microscope from smears of centrifuged deposits. Complement factors needed for phagocytosis were provided by pooled serum and the leukocyte was kept viable in Hank's Balanced Salt Solution.

MATERIALS

1. Leukocytes suspended in Hank's Balanced Salt Solution.
2. Hank's Balanced Salt Solution.
3. Candida albicans suspension.

Candida cells suspended in Hank's solution at a concentration of 5 $x10^6$ cells/ml. The yeasts were killed by heating at 100 ^{0} C for 30 mins. A large batch was thus prepared and divided into small aliquots sufficient for each test. These were stored at -20^0C until use.

4. Pooled Human serum
5. Phosphate Buffered Saline (PBS)

METHOD

A sets of test tubes which include 0.25ml Hank's solution, 0.25 ml pooled human serum, 0.25ml of heat killed Candida and 0.25ml leukocytes suspension were added. The test tubes were incubated at 37^0C for 30 minutes. Then they were centrifuged at 2000 RPM for 30 minutes and the supernatant was removed with a Pasteur pipette leaving a droplet into which the sediment was resuspended. Smears were made air dried and stained with Wright's stain. 100 neutrophils were examined and the number of neutrophils positive for

Candida ingestion counted. The number of Candida spores ingested per cell was also counted. Phagocytosis was thus expressed as the number of particle internalized per 100 neutrophils.

RESULT:

Normal neutrophils may contain anything from no Candida to five or more per cell. A normal control tested at the same time as the patient's cells and any difference is noted.

SPECIFIC GRANULE RELEASE ASSAY [71]

Specific granule release from stimulated human neutrophils was done using qualitative test by Nitro Blue Tetrazolium (NBT) test.

MATERIALS USED:

1. Phosphate Buffered Saline (PBS) P^H -7.2
2. Endotoxin (E.Coli-H sigma Chemicals, U.S.A.)
3. Heparin 150 to 200 Unit/ml of blood

The endotoxin is made up as a solution of 1mg/ml in PBS.

0.1ml of endotoxin is added to the NBT and heparinised blood mixture for stimulation of cells.

METHOD

2ml of venous blood was collected and 200 unit of Heparin added as an anticoagulant.6 drops of working NBT solution were added to 6 drops of Heparinised blood and gently mixed. The mixture was incubated at 37^0C for 30 mins. At the end of 30 minutes the solution was gently mixed and cover-slip smears were made. The smears were dried and stained with Wright's stain. The stained smear was then examined under oil immersion objective and neutrophils with ingested, blue or bluish-black formazan crystals were

counted. A total of 100 neutrophils were counted and the percentage of positive neutrophils noted. The test was mathematically expressed as the number of neutrophils out of 100 that were positive for NBT reduction.

RESULT:

Less than 10% of normal unstimulated neutrophils contain blue granules. Stimulated cells may be over 90% positive.

Photograph No. 1

Group I - Diabetic with Generalized Chronic Periodontitis

Photograph No. 2

Group II - Non diabetic with Generalized Chronic Periodontitis

Photograph No. 3

Group III - Generalized Aggressive Periodontitis

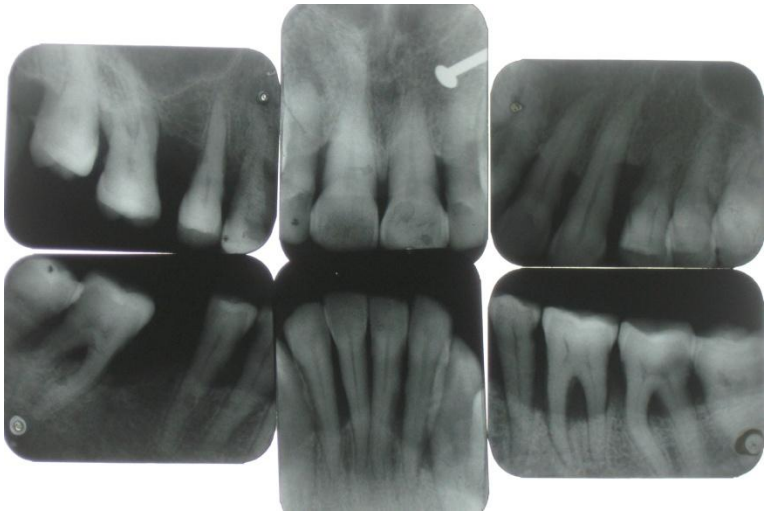
Photograph No. 4

Group IV - Control

Photograph No. 5

Measurements of probing depth with William's periodontal probe

Photograph No. 6

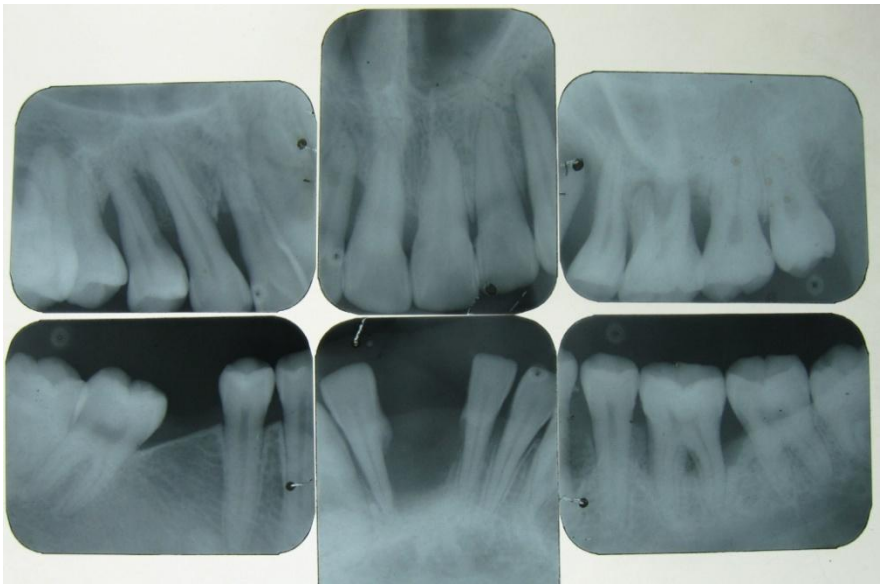


Photograph of Radiographs of Group I

Photograph No. 7

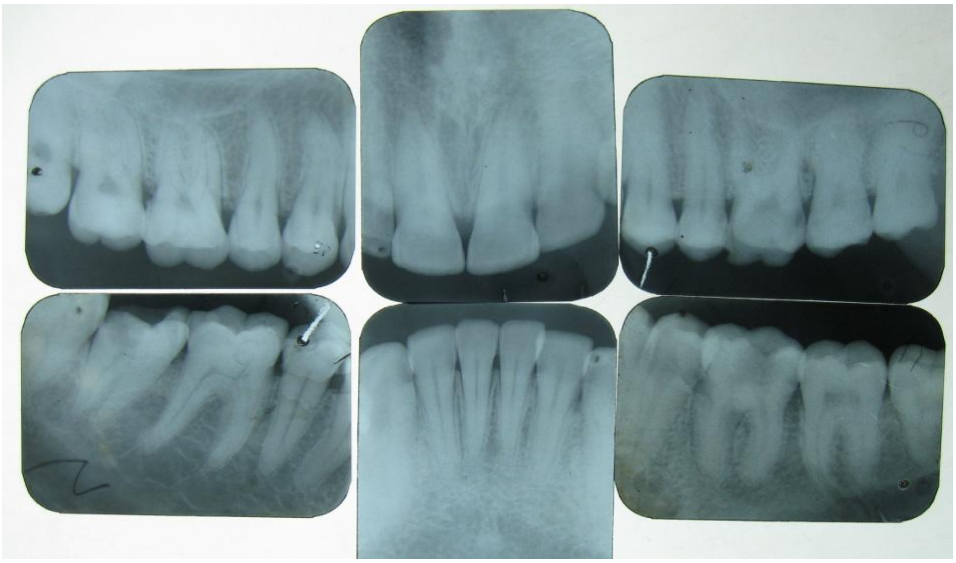
Photograph of Radiographs of Group II

Photograph No. 8



Photograph of Radiographs of Group III

Photograph No. 9



Photograph of Radiographs of Group IV

Photograph No. 10

Armamentarium used for periodontal examination and blood sample collection

Photograph No. 11

Collection of the venous blood sample

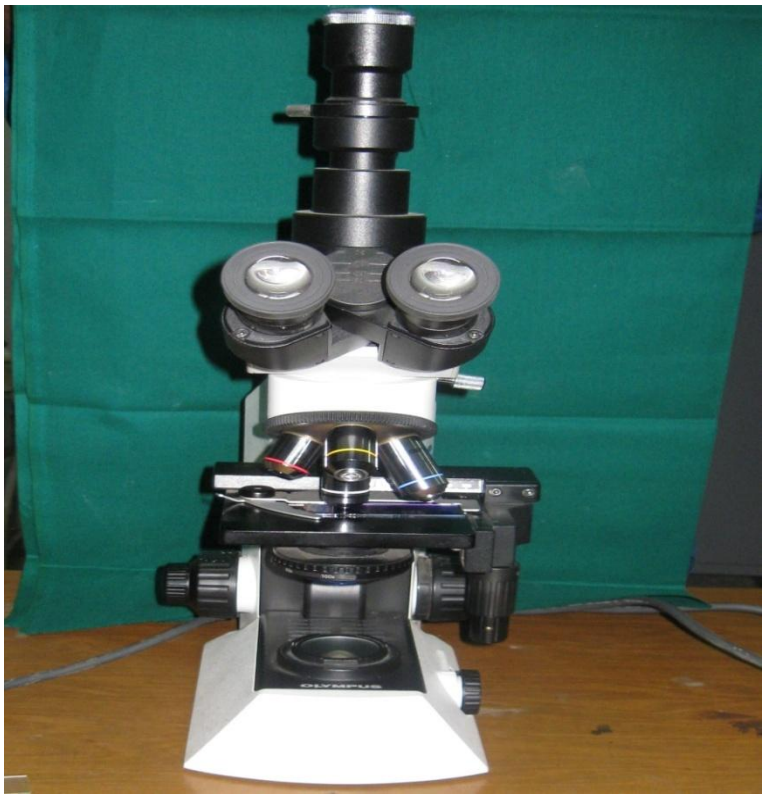
Photograph No. 12

Armamentarium for sample transportation

Photograph No. 13

Armamentarium used for neutrophil function test

Photograph No. 14



Microscope

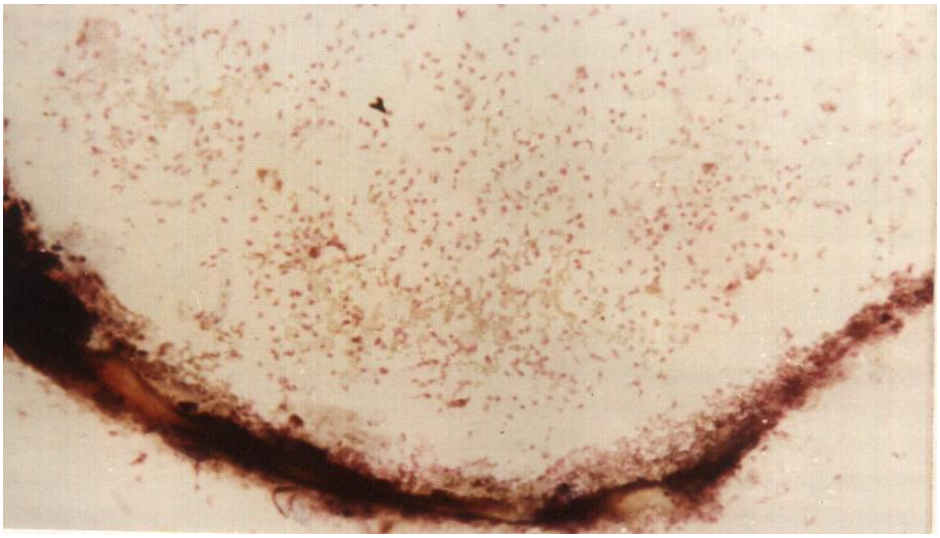
Photograph No. 15

Incubator

Photograph No. 16

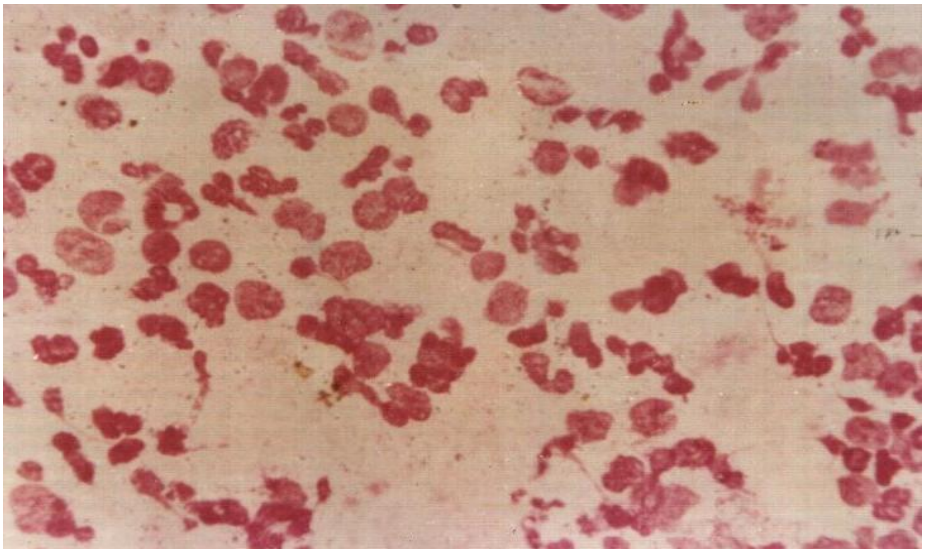
Centrifuge Machine

Photograph No. 17



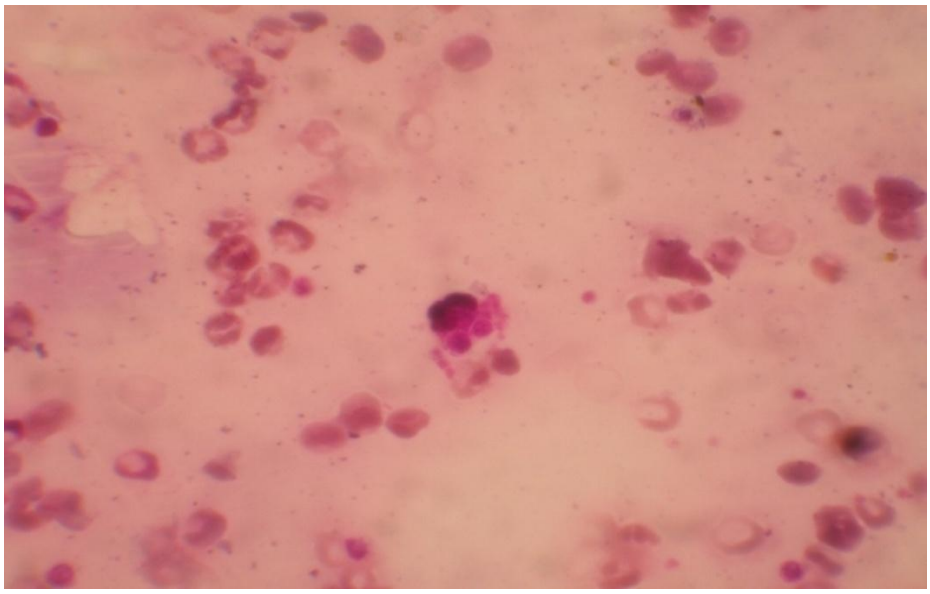
Photomicrograph showing neutrophil migrating towards chemoattractant- low power

Photograph No. 18



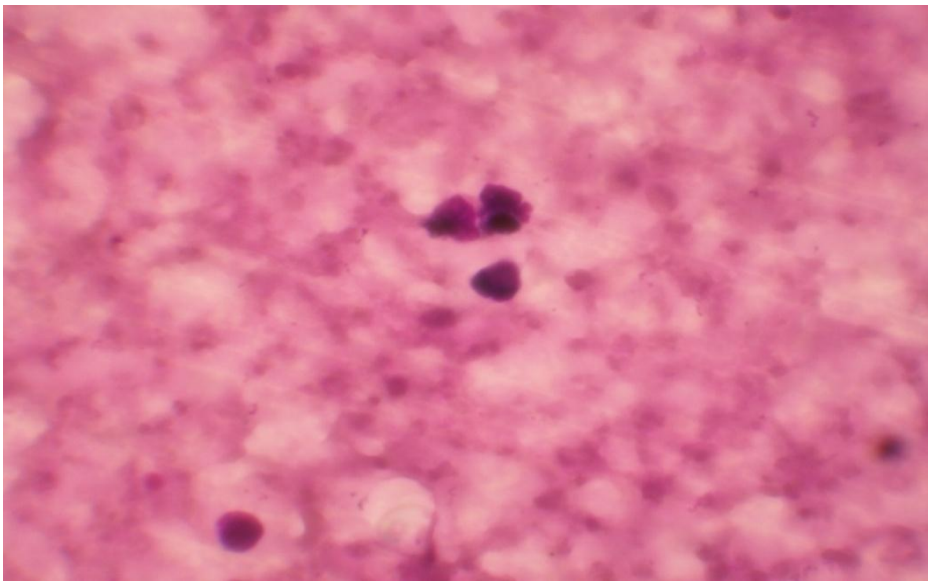
Photomicrograph showing neutrophil migrating towards chemoattractant- High power

Photograph No. 19



Photomicrograph showing neutrophil phagocytosis of candida

Photograph No. 20



Photomicrograph showing formazan crystal inside neutrophil (NBT Reduction)

STATISTICAL ANALYSIS

The statistical package SPSS PC + (Statistical Package for Social Science, Version 4.0.1) were used for statistical analysis.

Analysis of variance **(ANOVA) followed by Tukey – HSD** test was the technique used in this study to compare the mean values between control, study group A and B. ANOVA is used to test equality of means, when more than two populations are considered.

A set of N = Cr observations classified in one direction may be represented as follows:

$$\text{Mean} = \frac{X_{1r} \quad X_{2r} \quad X_{cr}}{\overline{X}_1 \quad \overline{X}_2 \quad \overline{X}_c}$$

$$\overline{X} = \frac{\overline{X}_1 + \overline{X}_2 + \ldots\ldots \overline{X}_c}{C}$$

Then

`Between column' sum of squares: SSC = $\sum_j \left(X_j - \overline{\overline{X}}\right)^2$

`Within column' sum of squares: SSE = $\sum_j \sum_i \left(X_{ij} - \overline{X_j}\right)^2$

Total sum of squares: SST = $\sum\sum \left(X_j - \overline{\overline{X}}\,\overline{j}\right)^2$

SST = SSC + SSE

Tukey's HSD Post Hoc Test Steps

FORMULA:

$$\frac{M_1 - M_2}{\sqrt{MS_w\left(\frac{1}{n}\right)}}$$

M = treatment/group mean
n = number per treatment/group

Steps

1. Calculate an analysis of variance (e.g., One-way between-subjects ANOVA).
2. Select two means and note the relevant variables (Means, Mean Square Within, and number per condition/group)
3. Calculate Tukey's test for each mean comparison
4. Check to see if Tukey's score is statistically significant with Tukey's probability/critical value table taking into account appropriate df within and number of treatments.

RESULT

RESULT

In the present study, 60 subjects were included, among them 15 Diabetic with Generalized Chronic Periodontitis were categorized as **Group** I, 15 Non Diabetic patient with Generalized Chronic Periodontitis were categorized as **Group II,** 15 Generalized Aggressive Periodontitis were categorized as **Group III,** and healthy control as **Group IV.** The clinical parameters used were plaque index, periodontal index, probing depth and clinical attachment loss.

The neutrophils function tests were performed under identical condition for the entire sample. The individual score for each test for each sample were calculated and finally, the mean values and standard deviation obtained for all three tests in all four groups.

The results were subjected to statistical analysis using **ANOVA** followed by **Tukey HSD.**

Table no.1 & Figure 1 Shows the comparison of mean and standard deviation of plaque index. It was found that plaque index was less in Group I and in Group III as compared to Group II with mean value of 2.273 ± 0.1533 for Group I, 2.800 ± 0.2181 for Group II, 1.353 ± 0.155 for Group III and 0 for Group IV with **p value <0.001** which is highly significant statistically.

Table no.2 & Figure 2 Shows the comparison of mean and standard deviation of periodontal index. It was found that periodontal index was more in Group I and in

Group III as compared to Group II with mean value of 4.166 ± 0.134 for Group I, 3.813 ± 0.130 for Group II, 6.126 ± 0.162 for Group III and 0 for Group IV with **p value <0.001** which is highly significant statistically.

Table no.3 & Figure 3 Shows the comparison of mean and standard deviation of neutrophil chemotaxis between four groups. It was found that neutrophil chemotaxis was defective in Group I and in Group III as compared to control and Group II with mean value was 1.340 ± 0.2028 for Group I, 2.747 ± 0.130 for Group II, 1.287 ± 0.135 , for Group III, and 2.76 ± 0.129 for Group IV, with **P<0.001** which was statistically highly significant.

Table no.4 Shows the multiple comparison of mean and standard deviation of neutrophil chemotaxis between four groups.

Table no.5 & Figure 4 Shows the comparison of mean and standard deviation of neutrophil phagocytosis between four groups. It was found that neutrophil phagocytosis was defective in Group I, Group II, and in Group III as compared to control with mean value of 187.33± 6.207, for group I, 281.40 ± 6.057,for group II , 277.13 ± 4.373 for Group III and 296 ± 4.367 for Group IV with **P>0.001** which was statistically significant.

Table no.6 Shows the multiple comparison of mean and standard deviation of neutrophil phagocytosis between four groups.

Table no.7 & Figure 5 Shows the comparison of mean and standard deviation of Specific Granules Release between four groups. It was found that granules release

was defective in Group I as compared to control with mean value 77.87 ± 2.295, for Group I, 80.13 ± 3.021 for Group II, 78. ± 2.449 for Group III and 81.87 ± 2.326 for Group IV with **P>0.001** which was statistically significant.

Table no.8 Shows the multiple comparison of mean and standard deviation of Specific Granules Release assay among four groups.

Note

P value

0 to 0.01 - ** significant at 1% level

0.011 to 0.05 - * significant at 5% level

> 0.05 not significant at 5% level

TABLE NO.1

COMPARISON OF MEAN & STANDARD DEVIATION OF PLAQUE INDEX BETWEEN FOUR GROUPS

Group	N	Mean	St Deviation	P Value
Group I	15	2.2733	.15337	< 0.001**
Group II	15	2.8000	.21381	
Group III	15	1.3533	.15523	
Group IV	15	.0000	.00000	

TABLE NO.2

COMPARISON OF MEAN & STANDARD DEVIATION OF PERIODONTAL INDEX BETWEEN FOUR GROUPS

Group	N	Mean	Std. Deviation	P Value
Group I	15	4.1667	.13452	< 0.001**
Group II	15	3.8133	.13020	
Group III	15	6.1267	.16242	
Group IV	15	.0000	.00000	

TABLE NO. 3

COMPARISON OF MEAN & STANDARD DEVIATION OF CHEMOTAXIS AMONG FOUR GROUPS

Group	N	Mean	Std. Deviation	P Value
Group I	15	1.340	.2028	<0.001**
Group II	15	2.747	.1302	
Group III	15	1.287	.1356	
Group IV	15	2.767	.1291	
Total	60	2.035	.7431	

TABLE NO. 4

MULTIPLE COMPARISONS OF MEAN & STANDARD DEVIATION OF CHEMOTAXIS BETWEEN FOUR GROUPS

Group	(J) Group	Mean Difference (I-J)	Std. Error	P value
Group I	Group II	-1.407(*)	.0557	<0.001**
	Group III	.053	.0557	<.774
	Group IV	-1.427(*)	.0557	<0.001**
Group II	Group I	1.407(*)	.0557	<0.001**
	Group III	1.460(*)	.0557	<0.001**
	Group IV	-.020	.0557	.984
Group III	Group I	-.053	.0557	.774
	Group II	-1.460(*)	.0557	<0.001**
	Group IV	-1.480(*)	.0557	<0.001**
Group IV	Group I	1.427(*)	.0557	<0.001**
	Group II	.020	.0557	.984
	Group III	1.480(*)	.0557	<0.001**

TABLE NO. 5

COMPARISONS OF MEAN & STANDARD DEVIATION OF PHAGOCYTOSIS BETWEEN FOUR GROUPS

Group	N	Mean	Std. Deviation	P value
Group I	15	187.33	6.207	<0.001**
Group II	15	281.40	6.057	
Group III	15	277.13	4.373	
Group IV	15	296.73	4.367	

TABLE NO. 6

MULTIPLE COMPARISONS OF MEAN & STANDARD DEVIATION OF PHAGOCYTOSIS BETWEEN FOUR GROUPS

Group	(J) Group	Mean Difference (I-J)	Std. Error	P value
Group I	Group II	-94.07(*)	1.944	<0.001**
	Group III	-89.80(*)	1.944	<0.001**
	Group IV	-109.40(*)	1.944	<0.001**
Group II	Group I	94.07(*)	1.944	<0.001**
	Group III	4.27	1.944	.137
	Group IV	-15.33(*)	1.944	<0.001**
Group III	Group I	89.80(*)	1.944	<0.001**
	Group II	-4.27	1.944	.137
	Group IV	-19.60(*)	1.944	<0.001**
Group IV	Group I	109.40(*)	1.944	<0.001**
	Group II	15.33(*)	1.944	<0.001**
	Group III	19.60(*)	1.944	<0.001**

TABLE NO. 7

COMPARISONS OF MEAN & STANDARD DEVIATION OF SPECIFIC GRANULE RELEASE ASSAY BETWEEN FOUR GROUPS

Group	N	Mean	Std. Deviation	P Value
Group I	15	77.87	2.295	< 0.001**
Group II	15	80.13	3.021	
Group III	15	78.00	2.449	
Group IV	15	81.87	2.326	

TABLE NO.8

MULTIPLE COMPARISONS OF MEAN & STANDARD DEVIATION OF SPECIFIC GRANULE RELEASE ASSAY BETWEEN FOUR GROUPS

Group	(J) Group	Mean Difference (I-J)	Std. Error	P value
Group I	Group II	-2.27	.927	.080
	Group III	-.13	.927	.999
	Group IV	-4.00(*)	.927	<0.001**
Group II	Group I	2.27	.927	.080
	Group III	2.13	.927	.110
	Group IV	-1.73	.927	.253
Group III	Group I	.13	.927	.999
	Group II	-2.13	.927	.110
	Group IV	-3.87(*)	.927	.001
Group IV	Group I	4.00(*)	.927	<0.001**
	Group II	1.73	.927	.253
	Group III	3.87(*)	.927	.001

Figure: 1

COMPARISON OF MEAN PLAQUE INDEX BETWEEN FOUR GROUPS

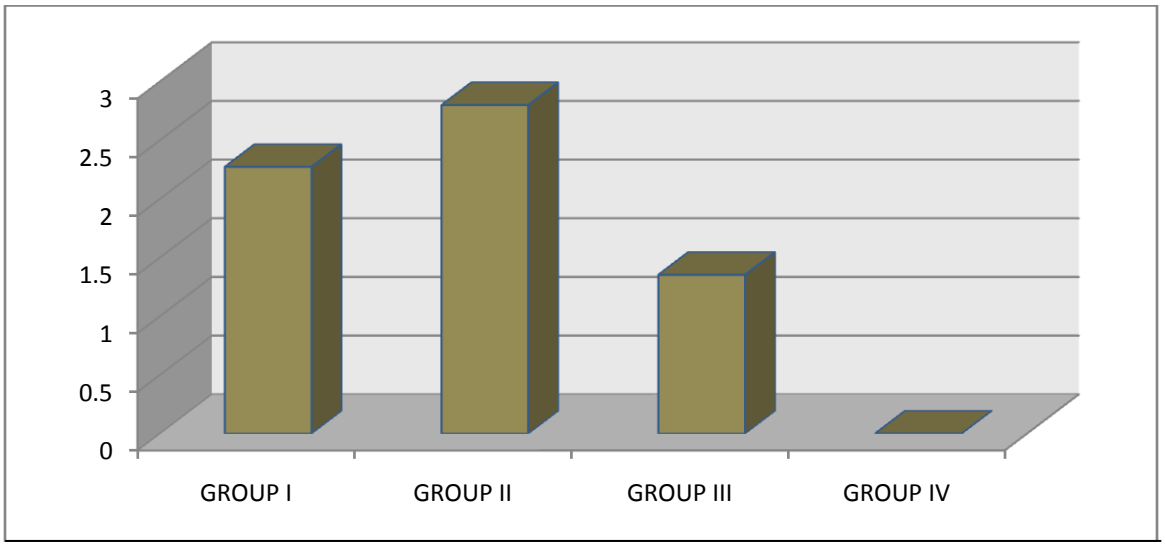


Figure: 2

COMPARISON OF MEAN PERIODONTAL INDEX BETWEEN FOUR GROUPS

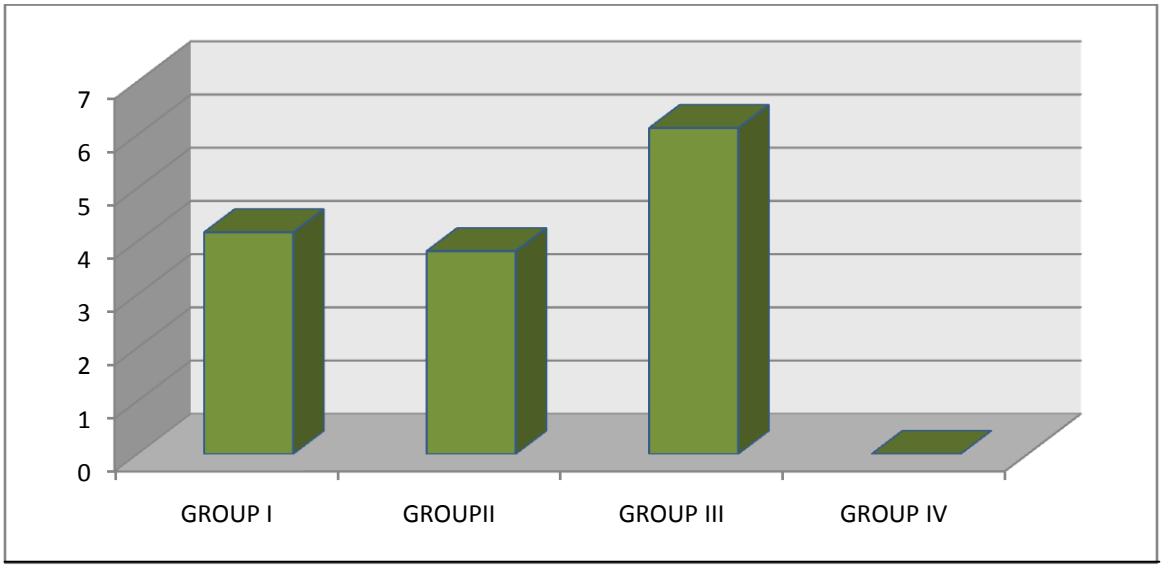


Figure -3

COMPARISON OF MEAN CHEMOTAXIS BETWEEN FOUR GROUPS

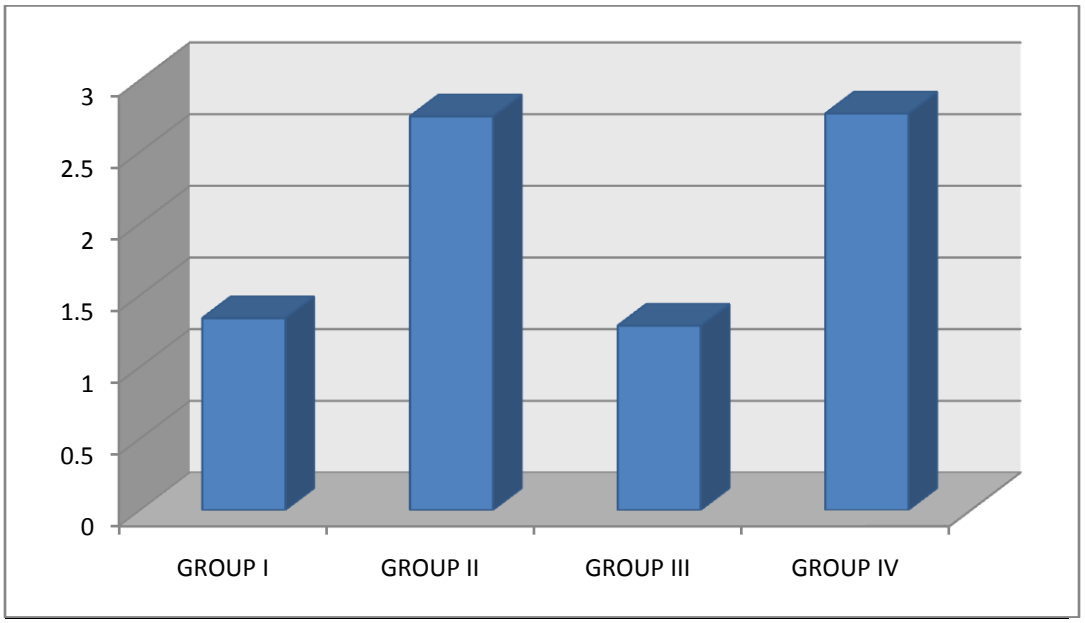


Figure - 4

COMPARISON OF MEAN PHAGOCYTOSIS BETWEEN FOUR GROUPS

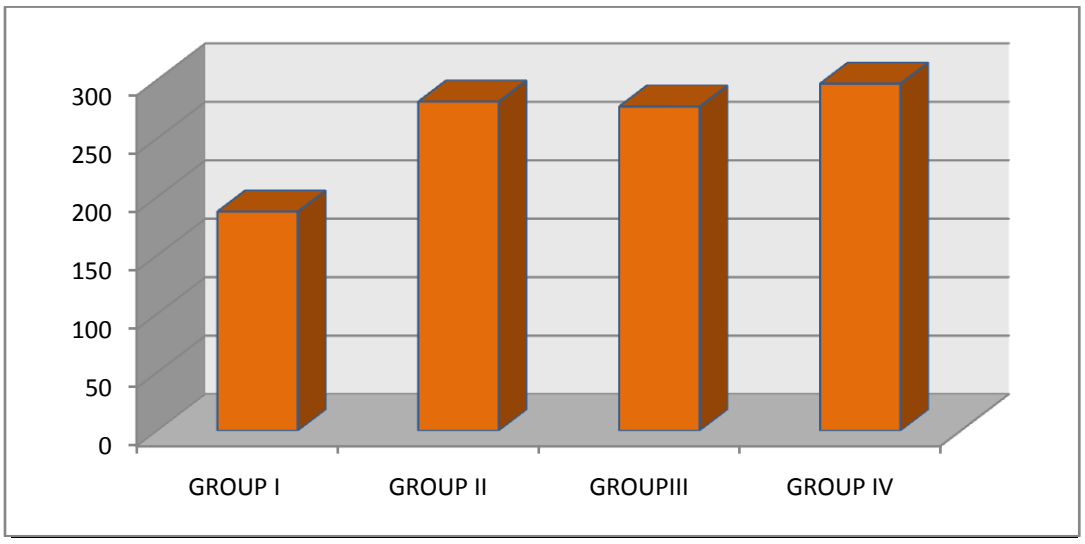
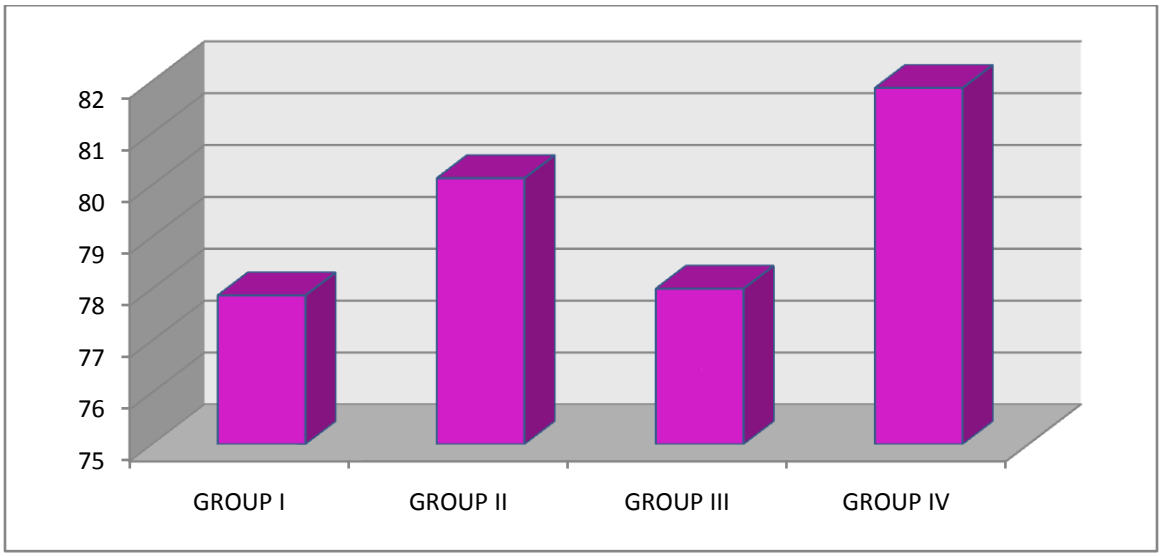


Figure- 5

COMPARISON OF MEAN SPECIFIC GRANULE RELEASE AMONG FOUR GROUPS



DISCUSSION

DISCUSSION

The probable influence of diabetes mellitus on the onset and duration of periodontal disease has been studied for many years. On the basis of experimental and clinical investigations, it is generally accepted that diabetes may result in a greater severity of periodontal disease. The mechanism of increased susceptibility to periodontitis in diabetics is not entirely clear. According to a few investigators, the primary factor responsible for the development of diabetic complications is prolonged tissue response to hyperglycemia, which results in the production of advanced glycated end products. Also, there may be an increase in the local production of cytokines leading to connective tissue damage, bone resorption and delayed wound repair. Several studies in diabetic humans have reported defects in neutrophil functions namely chemotaxis, phagocytosis, killing and microbicidal and super oxide released, which could be one of the reasons for increased susceptibility to Periodontitis in these subjects.[10, 18,,24 ,25,7]

Periodontitis is a disease attributable to multiple infectious agents and interconnected cellular and humoral host responses. It is widely accepted that bacteria or bacterial substances of the dental plaque are the primary etiologic factors in the initiation of inflammatory periodontal disease. The specific microflora composition in periodontal pocket is important in determining the severity and extent of destruction to the periodontium. However, an individual's susceptibility may be an additional and important modifying factor in the pathogenesis of periodontitis.[47]

In the last decade it has been established that increased susceptibility to certain periodontal diseases is associated with decreased leukocyte numbers and/or functional

capabilities. In addition, there appears to be an association between aggressive forms of periodontitis and certain hereditary disorders. Few studies have also shown the association of neutrophil functions in Chronic Periodontitis. Aggressive Periodontitis comprises a group of rare, often severe types of periodontal diseases that affect mainly young patients, occur in localized and generalized forms, have a rapid attachment loss and bone resorption, and are marked by familial aggregation. Chronic Periodontitis group, manifests a later onset, usually during the fourth or fifth decade of life, and has a much slower rate of progression.[3, 13]

Over the years, data supporting the microbial etiology of periodontal diseases have led to the recognition of several periodontally pathogenic species. PMNs form the first line of defense against these periodontal pathogens in the gingival sulcus. At this site, PMNs are actively involved in phagocytosing and killing bacteria through degranulation of enzymes and production of oxygen radicals. The phagocytosis of bacteria at this interface is enhanced dramatically by specific antibody recognition. There is clear evidence of the importance of this protective PMN barrier, as patients with abnormalities in the numbers and/or functions of these cells often experience rapid and severe periodontal destruction. PMN dysfunctions have also been associated with recurrence of disease after treatment. The concept that PMNs are important protective cells is further supported by the fact that many periodontal organisms possess virulence factors that suppress neutrophils activities. It remains unclear whether neutrophil impairment is an intrinsic characteristic in patients with severe destructive diseases. The current study aimed to assess neutrophil functions i.e. Chemotaxis, Phagocytosis and Specific Granule

Release in Diabetic and Non Diabetic patient with Generalized Chronic Periodontitis and in Generalized Aggressive Periodontitis patients.

The present study was carried out in 60 subjects comprising of both males and females reporting to the department of Periodontics in **Tamil Nadu Government Dental College and Hospital,** Chennai. The subjects were divided into four equal groups based on their periodontal status according to various clinical parameters which included plaque index, and periodontal index, probing depth, clinical attachment level and neutrophils functions that included Chemotaxis under agarose technique, Phagocytosis of killed Candida albicans and Specific Granule Released using Nitro Blue Tetrazolium Test (NBT).

In exclusion criteria of this study we took account of many factors that affects the neutrophils functions. We have excluded chronic smokers, tobacco users from our study as it may affect neutrophils chemotaxis. It is corroborates with the study done by Margereta et al 1999 who showed that smoking per se induces several changes in the blood increased radical changes in peripheral neutrophils, decreased chemotaxis, phagocytosis, imbalances between plasma proteases and oxidants.

Female patients on contraceptives, menstruation and pregnancy were excluded as it might have an effect on phagocytic activity of PMNs. The influence of gender on neutrophil functions remains uncertain; however, data using animal models indicated that sexual hormones seem to have different effects on chemotactic, phagocytic activities of PMNs.[40]

When the periodontal status of Group IV(periodontally healthy subjects) was compared with that Group I (Diabetic with Generalized Chronic Periodontitis) Group II(

Non Diabetic with Generalized Chronic Periodontitis) and Group III (Generalized Aggressive periodontitis) subjects , the amount of local factors was less and periodontal destruction was more in Group I and Group III subjects as compared to Groups II. The mean plaque score for Group I was 2.273 ± 0.1533 and for Group III 1.353 ± 0.155 which was significantly less in Group I and in Group III than Group II which was 2.800 ± 0.218. This finding in our study was in agreement with studies done by **Listgarten et al** [42] who reported a significantly less plaque plaque score in diabetics and also with **Van Dyke et al**, **Offenbacher et al** [64] who reported a significantly less plaque score in GAgP subjects. The mean periodontal index score 6.126 ± 0.162 was significantly more in Group III than Group I which was 4.166 ± 0.134 and Group II which was 3.813 ± 0.130. This finding in our study was in agreement with **Heikki Repo et al** [27]**; Van Dyke et al; Offenbacher et al** [64] who showed that mean periodontal index score was more in GAgP patient. Our findings were compatible with the expected clinical findings of patients with aggressive periodontitis.

The present study evaluated the comparison between peripheral PMN chemotaxis, phagocytosis and specific granule release among four groups. Our data showed that in Group I and Group III subjects, most exhibited defective chemotaxis towards FMLP chemoattractant as compared to Group II and Group IV (periodontally healthy control subjects) (fig). And good chemotaxis towards FMLP was observed in Group II and Group IV subjects with periodontally healthy subjects (fig). The mean chemotaxis between FMLP of Group I was 1.340 ± 0.2028, group II was 2.747 ± 0.130 Group III was 1.287± 0.135 and 2.767 ± 0.129 for Group IV with **P<0.001** which was statistically highly significant. These results extend and confirm earlier studies in which defective

PMNL chemotaxis was reported in GAgP patient by **Cianciola et al** [62], by **Clark et al** [14] and by **Lavine et al** [37]**.** Our study also in agreement with study done by **Christopher W Cutler et al** [19]

It has become apparent in recent years that GAgP subjects are not a totally homogenous group with regard to neutrophils function. Many laboratories agree that neutrophils chemotaxis is depressed in 65-75% of GAgP patients. However reports of population of GAgP patients with no detectable neutrophils abnormalities also appear. The most likely explanation for these discrepancies is population differences, in that the groups without abnormalities were genetically homogenous. Since GAgP subjects exhibit a geographic variation we carried out this study in Indian population and confirmed the chemotaxis defect in such patients.

The chemotaxis defect in Group II subjects was less as compared to GAgP subjects and was similar as compared to periodontally healthy subjects and was not statistically significant. Whereas results showed that chemotaxis was defective in Group III (GAgP) patients as compared to Group II and periodontally healthy subjects and was highly significant statistically. This proved that chemotaxis was not significantly defective in Group II subjects like GAgP subjects. This finding is in agreement with **Tufano et al 1992.**[67]

An interesting aspect of patients with GAgP is that they appear to be otherwise healthy. Nevertheless, about 75% of aggressive periodontitis patients have shown to have abnormal leukocyte chemotactic behavior. During the last decade, however, it has become increasingly clear that many patients with Aggressive Periodontitis may either fail to display any leukocyte chemotactic defect or their cells may actually display

enhanced chemotactic ability. There are three main sources of variability which may explain these discrepancies are:

1. Variations in clinical diagnostic criteria

2. Individual variability

3. Technical variation

Several methods are available for studying cell locomotory behaviour, of which the two most common are Boyden chamber technique and the under agarose migration method Chemiluminescence (CL) is also one of the methods by which neutrophils functions can be evaluated. In the Boyden chamber technique cells penetrate a porous filter and they are to some extent contact guided to some extent by the pores themselves. Under agarose migration method, the cells move on a 2 dimensional surface in the junctional plane between a plastic tissue culture dish and an overlaying agarose gel.[41, 46]. Chemiluminiscence is the light energy produced by the PMN during its interaction with bacteria or other particles. The method used in this study was under agarose migration method due to its relatively simple and practical system to use, in that it requires no special equipment like in Boyden chamber or Chemiluminescence and it uses low numbers of cells and small amounts of chemoattractant substances.

The present study also evaluated the comparison between peripheral PMN phagocytosis using killed Candida albicans among four groups. Our data revealed that Group I, Group II, and Group III patients showed significant difference in phagocytosis rate as compared to Group IV (periodontally healthy control group). The mean phagocytosis between group I was 187.33± 6.207, in group II was 281.40 ± 6.057, group III was 277.13 ± 4.373, and296 ± 4.367with P>0.001 which was statistically significant.

Our finding was in agreement with studies of **Cianciola et al 1977**,[62] **Van Dyke et al 1986,**[64] Kimura et al 1992 Eick et al 2000 who showed that GAgP patients presented a significantly diminished phagocytosis rate of opsonised bacteria compared with periodontally healthy subjects. Our finding also in agreement with study by **Cutler et al** [19] who found impaired phagocytosis in diabetic patient.

When specific granule release was assayed using stimulated cells in the presence of Nitro Blue Tetrazolium (NBT) dye, it was found that stimulated cells from all the three groups showed that the dye was taken up into phagosomes and intracellular reduction of dye converted it into an insoluble blue crystalline form which was visible in light microscope. The NBT stimulated for Group I was 77.87 ± 2.295, for Group II was 80.13 ± 3.021 for group III was 78. ± 2.449 and for Group IV was 81.87 ± 2.326 with P>0.001which was statistically significant.

This suggested that Group I subjects were less able to reduce NBT as compared to healthy controls. This is in agreement with the study done by **Veta et al 1993** [69]**.** But when Group II, and Group III subject were compare with group IV the values for the test group did not differ significantly from the control group. This shows that the neutrophils in this condition are not metabolically defective. Our observations are in agreement with the study done by **Shurine et al** [61] in 1979 and **Zafiropoulas in 1988**.[71] and also with **Van Dyke et al in 1986.**[64]

Conflicting data among the present study and other studies may have resulted from differences in study population, assays for chemotaxis, phagocytosis, specific granule release detection. One possible limitation of our study may have been the small size of the sample population. Another aspect that should be considered in this study is related to

the criteria used for the diagnosis of periodontal disease or health. This has been a controversial issue in many clinical and microbiological studies in Periodontology. There is a consensus that the classification and diagnosis of diseases such as periodontitis should be based on clinical, environmental and laboratory parameters related to host microbial interactions. However, at the moment the definition of periodontal health or disease continue to rely on well described clinical criteria that the level of disease of a particular population.[3, 13]

Thus it can be suggested that the assessment of neutrophils functions viz, chemotaxis, phagocytosis rate, specific granule release may be an important and useful tool for explaining various types of periodontal pathologies. Defective neutrophil function may play an important role in the induction and pathogenesis of severe periodontitis in young individuals. Disease resulting in destruction of periodontal support such as seen in aggressive periodontitis may be due to decreased capabilities of selected aspects of neutrophils function.

SUMMARY AND CONCLUSION

SUMMARY & CONCLUSION

Periodontal diseases have a multifactorial aetiology and microbial infections play a prominent role in the pathogenic process. Neutrophils are the key cellular elements of the innate immune system, providing protection from invading bacteria. Functional defects in peripheral PMNs have also been implicated in the pathogenesis of Periodontitis. Evidence for the protective function of PMNs in periodontal disease comes from the observations that individuals with severe periodontitis exhibit quantitative and/ or qualitative defects in their peripheral PMNs. [1, 30]

The aim of the present study was to assess Neutrophil functions i.e. Chemotaxis, Phagocytosis and Specific Granule Release in patients with diabetic and non diabetic patients with generalized chronic periodontitis and in generalized Aggressive Periodontitis in Indian population. 60 patients were selected to participate in the study which was divided into four groups. These patients were selected according to clinical and radiographic criteria. Blood samples from 60 patients were collected after drawing 5ml peripheral venous blood and then various assays were carried out to assess the neutrophils functions mentioned above. Statistical analysis of the results was done using **ANOVA** followed by **Tukey HSD** test.

Following **conclusions** were drawn from the present study:

1. Statistically highly significant differences were found in the Chemotaxis of Diabetic with Generalized Chronic Periodontitis and in Generalized Aggressive Periodontitis subjects as compared to Non Diabetic with Generalized Chronic Periodontitis subjects and periodontally healthy control subjects.
2. Statistically highly significant difference was observed in the Phagocytosis rate of Diabetic and Non Diabetic with Generalized Chronic Periodontitis and in Generalized Aggressive Periodontitis subjects, when compared to periodontally healthy control subjects.
3. A statistically highly significant difference between the Specific Granule Release using stimulated Nitro Blue Tetrazolium test were observed in Diabetic with Generalized Chronic Periodontitis subjects when compared to periodontally healthy control subjects.

Although, these findings do favour that defects or diminished activity in PMNs are also one of the factors that are responsible for the pathogenesis of periodontal diseases, and also a possible mechanism to render diabetic patients are more susceptible to periodontal diseases.[24] Further longitudinal and clinical trials with larger sample size and newer sensitive techniques are needed.

To summarise, in the present study the chemotaxis and phagocytic defect was detected in diabetic and GAgP subjects which showed that there was a cellular intrinsic defect in such subjects and also significant differences in reduction in specific granule release using qualitative stimulated NBT was detected in diabetic subject which showed that normal microbicidal activity was reduced in infection and intracellular killing

capacity of neutrophils was reduced. The important role of PMNs play in optimal functioning of the immune defence system has led to speculation that a partly compromised system could severely weaken the defence mounted against a bacterial insult and permit the occurrence and progression of infections and play an important role in induction and pathogenesis of periodontal diseases.[17] The role of neutrophils functions in the aetiology and pathogenesis of destructive periodontal disease merits further investigation.

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APPENDIX

DEPARTMENT OF PERIODONTICS

TAMILNADU GOVERNMENT DENTAL COLLEGE AND HOSPITAL, CHENNAI 600003.

Comparison of neutrophil function in diabetic and non diabetic patients with generalized chronic periodontitis and in generalized aggressive periodontitis patients and to compare this study in healthy control group.

PROFORMA

Date: Dental O.P. No: Code No:

Name: Age / Sex:

Address: Tel. no: Mobile no:

Occupation: Income:

Chief Complaints:

Pain / Shaky teeth / Bleeding gums / Swollen Gums / Receding Gums / Pus Discharge / Increase in Spacing between teeth / Stains / Others.

Duration:

Medical history:

1. Diabetes Mellitus
2. Pregnancy / Lactation
3. Cardiac diseases

4. Stroke
5. Any drugs against hypercholesterolemia
6. Liver dysfunction

Dental history:

Periodontal treatment within past 6 months.

Clinical Examination:

PLAQUE INDEX – SILNESS AND LOE (1964)

18	**17**	**16**	**15**	**14**	**13**	**12**	**11**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
48	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**

Calculation =

Inference =

PERIODONTAL INDEX: (RUSSELL, 1956)

18	**17**	**16**	**15**	**14**	**13**	**12**	**11**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
48	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**

PI =

PROBING DEPTH (PD) AND CLINICAL ATTACHMENT LEVEL (CAL)

MAXILLARY:

Palatal

CAL																
PPD																
	18	**17**	**16**	**15**	**14**	**13**	**12**	**11**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
PPD																
CAL																

Buccal

MANDIBULAR:

Lingual

CAL																
PPD																
	48	**47**	**46**	**45**	**44**	**43**	**42**	**41**	**31**	**32**	**33**	**34**	**35**	**36**	**37**	**38**
PPD																
CAL																

Buccal

DIAGNOSIS

Sample details:

Site:

Date:

INVESTIGATION:
IOPA X- ray
OPG

RADIOGRAPHIC FINDINGS:

DISTRIBUTION:

PATTERN OF BONE DESTRUCTION:

NEUTROPHIL FUNCTION TEST:

- CHEMOTAXIS
- PHAGOCYTOSIS
- SPECIFIC GRANULE RELEASE ASSAY

INFERENCE:

Signature of P.G student

Date:
Time:

INFORMED CONSENT FORM

STUDY TITLE:

COMPARISON OF NEUTROPHIL FUNCTIONS IN DIABETIC AND NON DIABETIC WITH GENERALIZED CHRONIC PERIODONTITIS AND IN GENERALIZED AGGRESSIVE PERIODONTITIS PATIENTS AND TO COMPARE THIS STUDY IN HEALTHY CONTROL GROUP.

Name: O.P.No:

Address: Code No:

Age / Sex:

I,_____age____years exercising my free power of choice, hereby give my consent to be included as a participant in the study **“” comparison of Neutrophil function in diabetic and non diabetic patient with generalized chronic periodontitis and in generalized aggressive periodontitis patients and to compare this study in control group .**
I agree to the following:

- I have been informed to my satisfaction about the purpose of the study and study procedures including investigations to monitor and safeguard my body function.
- I understand that the lab investigations will require the procurement of my blood in required amount.
- I agree to cooperate fully and to inform my doctor immediately if I suffer any unusual symptom.
- I have informed the doctor about all medications I have taken in the recent past and those I am currently taking.
- I hereby give permission to use my medical records for research purpose. I am told that the investigating doctor and institution will keep my identity confidential.

Name of the patient Signature / Thumb impression

Name of the investigator Signature Date